2271

β-Lactoglobulin variation in milk from individual cows

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SUMMARY. The β-lactoglobulin types of 812 individual cows of various breeds were determined by paper and polyacrylamide gel electrophoresis. The results support the hypothesis that β-lactoglobulins A and B are controlled genetically by codominant autosomal alleles (Lg^A and Lg^B).

Milks from 5 heterozygous cows (Lg^A/Lg^B) were examined by free electrophoresis and column chromatography to estimate the relative quantities of β-A and β-B produced by these animals. The results indicate that β-A and β-B are produced in approximately equal amounts, with β-A being slightly in excess of β-B.

Lacteal secretions were obtained from 2 castrated males and typed for β-lactoglobulin by means of paper electrophoresis. These animals were found to produce β-lactoglobulins (B and AB) consistent with their expected genotypes as deduced from pedigree analysis.

In 1955 Aschaffenburg & Drewry discovered the existence of 2 electrophoretically distinguishable forms of β-lactoglobulin, β-A and β-B, in cow's milk. After studying the β-lactoglobulins produced by individual animals, they reported that the observed variation was genetically controlled by 2 autosomal alleles with no dominance. This genetic hypothesis has been confirmed by workers in our group (Plowman, Townend, Kiddy & Timasheff, 1959), by Moustgaard, Møller & Havskov Sørensen (1960), and by Bell (1962) who discovered a 3rd allele producing β-lactoglobulin C which moves even slower than β-B in electrophoresis.

This paper presents further extensive data confirming the genetic hypothesis. It also presents the results of studies on the relative production of β-A and β-B by heterozygous animals, and on the occurrence of β-lactoglobulins in lacteal secretions from hormone-treated castrated males.

EXPERIMENTAL

The sample for electrophoresis was prepared from the portion of the whey soluble in 20% (w/v) Na_2SO_4; it was dialysed free of sulphate and concentrated tenfold by pervaporation. The typing work was initially done by paper electrophoresis using a modified technique of Aschaffenburg & Drewry (1955).

Later, vertical polyacrylamide gel electrophoresis was used for typing and is now used routinely (Peterson, 1963); it may be done directly on skim-milk (Aschaffenburg, 1964). Buffer was pH 8·6 veronal, ionic strength 0·025 and the polyacrylamide gel 50 g/l (Cyanogum 41†). One ml of skim-milk was diluted with 1 ml of the buffer and 0·3 ml of amido black (0·2 % solution in 60 % ethanol) was added as an indicator dye. Sucrose (about 150 mg) was added to increase the density and 20 μl of the sample mixture was applied to the gel. Electrophoresis was for 4 h at 60–65 mA (about 200 V) with circulation of cooling water at 10–15 °C.

Tiselius electrophoresis experiments were performed at 1 °C in a Spinco Model H† apparatus. Column chromatography was performed at room temperature using carboxymethylcellulose (CMC) and diethylaminoethylcellulose (DEAE) resins.

RESULTS AND DISCUSSION

Plate 1 (*a*) shows the results of electrophoresis in polyacrylamide gel of crystalline preparations of β-A, B, and C and their combinations in pairs. Plate 1 (*b*) is a photograph of a pattern obtained with skim-milk in routine typing. The β-lactoglobulin types of 812 cows of various breeds are shown in Table 1; data for 145 cows, from a

Table 1. *Distribution of β-lactoglobulin types by breeds*

Breed		Number of cows				χ^2	P	Gene frequency	
		Total	A	AB	B			A	B
Ayrshire	Observed	27	0	7	20	0·60	0·43	0·13	0·87
	Expected		0·5	6·1	20·5				
Brown Swiss	Observed	24	2	9	13	0·07	0·83	0·27	0·73
	Expected		1·8	9·5	12·7				
Guernsey	Observed	200	20	79	101	0·61	0·43	0·30	0·70
	Expected		18·0	84·0	98·0				
Holstein	Observed	406	86	205	115	0·09	0·77	0·46	0·54
	Expected		87·3	201·8	116·6				
Jersey	Observed	39	7	18	14	0·08	0·80	0·41	0·59
	Expected		6·6	18·9	13·6				
Cross-breds	Observed	116	2	58	56	8·82	0·003	0·27	0·73
	Expected		8·2	45·4	62·3				
Over-all	Observed	812	117	376	319	0·13	0·72	0·38	0·62
	Expected		114·5	380·8	316·7				

previous preliminary report (Plowman *et al.* 1959), have been included. For each of the pure breed groups, as well as the over-all group of 812, the distribution of the genotypes agrees well with that expected, assuming control by codominant autosomal alleles and random mating (P ranges from 0·35 to 0·83). In the cross-bred group, however, the difference between the observed and expected numbers of the various genotypes is highly significant ($P \simeq 0{\cdot}003$). The reason for this is that the random mating assumption is not valid for the cross-bred group. Of the 48 sires whose daughters were represented in the group of 116 crossbreds, 4 had 57 of the daughters in the group and these sires (2 Ayrshire and 2 Brown Swiss) were of the genotype

† It is not implied that the U.S. Department of Agriculture recommends the above companies or their products to the possible exclusion of others in the same business.

Lg^B/Lg^B. The average size of the other 44 sire groups was 1·3 daughters. Chi-square values for goodness-of-fit with the Hardy–Weinberg expectations were 7·9 ($P \simeq 0{\cdot}005$ for the 57 daughters of the 4 Lg^B/Lg^B sires and 1·9 ($P \simeq 0{\cdot}16$) for the remaining 59 cows in the cross-bred group.

The segregation data in Table 2 provide further evidence to support the genetic hypothesis for the control of the β-lactoglobulin variants. The agreement with the expected results is very good as it has been in other studies (Aschaffenburg & Drewry, 1957*b*; Moustgaard *et al.* 1960).

Table 2. *Segregation of β-lactoglobulin alleles in female offspring from certain mating combinations*

Mating types		Offspring by β-lactoglobulin genotype A/A*	A/B	B/B
A/A × A/A	Observed	10	0	0
	Expected	10	0	0
A/A × A/B	Observed	26	25	0
	Expected	25·5	25·5	0
A/A × B/B	Observed	0	45	0
	Expected	0	45	0
A/B × B/B	Observed	0	43	49
	Expected	0	46	46
A/B × A/B	Observed	14	38	13
	Expected	16·25	32·5	16·25
B/B × B/B	Observed	0	0	19
	Expected	0	0	19

* Shortened form for Lg^A/Lg^A.

With the paper electrophoresis technique the β-C variant cannot be detected as its mobility, under these conditions, is essentially that of β-B. Therefore, the earlier data (about 300 animals) may have an occasional β-C animal listed as β-B. The chances of this are small, however, since most of the animals involved were Holstein–Friesians and the occurrence of β-C has not been reported in this breed. Furthermore, the Lg^C allele appears to be rare in the 2 breeds in which it has been found (Bell, 1962). Bell reported the occurrence of C in 24 of 135 Jersey cows and one of 85 Australian Illawarra Shorthorns. No gene frequencies were given, but a minimum figure for Lg^C in Bell's Jersey sample would be 0·09 (if all 24 animals were heterozygotes and assuming no technical error such as the acknowledged one which led to the report that A, B and C could be found in milk from an individual cow). Since Bell's report, a few heterozygous Lg^C animals and 2 homozygotes have been found among Jerseys and Jersey–Brahman crosses in the United States (Kalan & Thompson, personal communication). It remains to be seen if Lg^C is present to any appreciable extent in breeds other than Jersey.

A summary of gene frequency data reported by various workers is shown in Table 3. It appears that Lg^B is definitely the most common allele in all but the Holstein–Friesian (0·55 Lg^B) and Jersey (0·47 Lg^B) breeds. There is a possibility, as stated above, that the figure for Lg^B in the Jersey breed may be slightly inflated as a result of failure to distinguish between B and C in the early stages of the present study.

The information summarized in Table 3 does not indicate much similarity between related breeds. It must be remembered, however, that the Red Dane samples were not random ones (Moustgaard *et al.* 1960); this is also probably true of most of the other breed samples. Because of this and the small number of animals sampled in some breeds and locations, many of these gene frequency figures should be considered as preliminary estimates.

Table 3. *Summary of β-lactoglobulin gene frequencies in various breeds of cattle*

Breed	No. of cows	Gene frequencies A	Gene frequencies B	Location and reference
Ayrshire	27	0·13	0·87	United States*
	54	0·31	0·69	Great Britain (Aschaffenburg & Drewry, 1957*b*)
Brown Swiss	24	0·27	0·73	United States*
Campine	66	0·33	0·67	Belgium (Préaux & Lontie, 1961)
Guernsey	200	0·30	0·70	United States*
	27	0·22	0·78	Great Britain (Aschaffenburg & Drewry, 1957*b*)
Holstein–Friesian	406	0·46	0·54	United States*
	161	0·61	0·39	Denmark (Moustgaard *et al.* 1960)
	87	0·40	0·60	Great Britain (Aschaffenburg & Drewry, 1957*b*)
	1056	0·43	0·57	Germany (Comberg, Meyer & Gröning, 1964)
Icelandic Cattle	52	0·34	0·66	Iceland (Blumberg & Tombs, 1958)
Jersey	39	0·41	0·59	United States*
	158	0·56	0·44	Denmark (Moustgaard *et al.* 1960)
Shorthorn	87	0·11	0·89	Great Britain (Aschaffenburg & Drewry, 1957*b*)
Red Dane	293	0·14	0·86	Denmark (Moustgaard *et al.* 1960)
White Fulani	58	0·21	0·79	Nigeria (Blumberg & Tombs, 1958)
Zebu	138	0·09	0·91	India (Bhattacharya, Roychoudhury, Sinha & Sen, 1963)

* Extracted from Table 1.

Lacteal secretions of castrated males

As a check on the presumption that males would express their β-lactoglobulin genotype in lacteal secretions, 2 castrated males were treated with oestrogen and progesterone. Treatment consisted of subcutaneous injections of the hormones in corn oil, 100 mg/day of progesterone for 4 days, alternating with 20 mg/day of diethylstilbesterol for 4 days. Milk was obtained from the first animal, 4120, after 6 weeks on this regimen. This male had been castrated at the age of 2 months and hormone injections were started at 1 year of age. Milk was not obtained from the 2nd male, 4104, until 3 months of treatment had elapsed, and then in smaller quantities than from 4120. This animal had been castrated at 18 months of age and hormone treatment began 2 months later. One to three ml of apparently normal milk was obtained each day from 4120 and was stored at -10 to -15 °C. The average composition of four 20 ml pooled samples was 12·8 % total solids, 3·2 % milk fat, and 3·9 % protein. Thus, the milk seemed to be essentially normal for these components.

The ultracentrifugal pattern given by the whey (pH 6·5, phosphate buffer) could not be distinguished from that given by normal cow's milk. Tiselius electrophoresis of

the whey in pH 8·63 veronal buffer, 0·05 ionic strength, also gave a pattern very similar to that of normal milk.

For the typing experiments 23 ml of the milk was worked up following the procedure of Aschaffenburg & Drewry (1957*a*). Paper electrophoresis on the pervaporated Na_2SO_4 whey (Plate 2(*b*)) gave a pattern essentially identical with that given by the milk of an A/B cow (Plate 2(*a*)) with a few extra bands running slower than α-lactalbumin, and an unmistakable A/B β-lactoglobulin. This result was as expected since the dam of 4120 was A/A and the sire's type had been deduced as B/B.

The milk production of 4104 was very poor, averaging about 1 ml/day. A sample of the milk was freed of casein and fat by centrifugation and acid precipitation at pH 4·4. After dialysis of the whey against water and pervaporation, a paper electrophoresis run was made using whey from a known A/B cow prepared in the same manner as a control. These strips are shown in Plate 2(*c*) and 2(*d*). The milk can be seen to be rich in components moving more slowly than α-lactalbumin and in the range of mobilities known to be possessed by the immune globulins of milk (Larson & Jenness, 1955). The β-lactoglobulin band is single, however, and occurs at the position of β-B. Again, this was as expected since 4104's sire and dam were both B/B.

From these experiments it is concluded that the milk produced by such artificially stimulated mammary tissue in bovine males does express the β-lactoglobulin phenotype characteristic of the genotype deduced by pedigree analysis.

Table 4. *Compositional changes during β-lactoglobulin preparation*

Sample	Concentration, g/l	% β-B*
$(NH_4)_2SO_4$ ppt.	2·2	0·51
First supernatant	13·1	0·49
Second supernatant	7·6	0·60
Second crystal crop	12·6	0·36

* Electrophoretic analysis at pH 5·6 (Timasheff & Townend, 1960).

Relative fractions of β-A and β-B in AB milk

Crystallization of the mixed β-lactoglobulin and subsequent electrophoretic determination of its composition (Timasheff & Townend, 1960) was not the method of choice for determination of the relative amounts of β-A and β-B synthesized by a heterozygous animal. Observation has shown that β-A by itself forms crystals much more readily than β-B. As a result of this, crystallization of the mixture might well cause fractionation, as has been observed by Ogston & Tombs (1957), and a non-uniformity of the crystals forming at the beginning and end of crystallization. This was checked in a simple experiment as follows. An 800-ml sample of whey from an A/B animal (X-617) was carried through the Aschaffenburg & Drewry (1957*a*) preparation procedure and samples were taken of the ammonium sulphate-β-lactoglobulin precipitate, the first supernatant after crystals had just begun to form (a single overnight dialysis), and the supernatant and a part of the crystal crop after 4 days dialysis with three changes of dialysate. The β-lactoglobulin B contents of these fractions are given in Table 4.

It can be seen that some fractionation does occur, and the effect of this on the final composition might well be a function of the volume of the last crystallizing

solution, and other factors generally not reported. Other workers (Johannsen, 1958; Yaguchi, Tarassuk & Hunziker, 1961) have reported separation of whey protein fractions using chromatographic methods, and an attempt was made to apply quantitatively the ion exchange cellulose resins of Sober & Peterson (1960) to this system. Preliminary experiments using gradients of ionic strength at fixed pH on DEAE or CMC were not satisfactory in separating the numerous components of milk whey (Brunner *et al.* 1960), although under other conditions (Yaguchi *et al.* 1961;

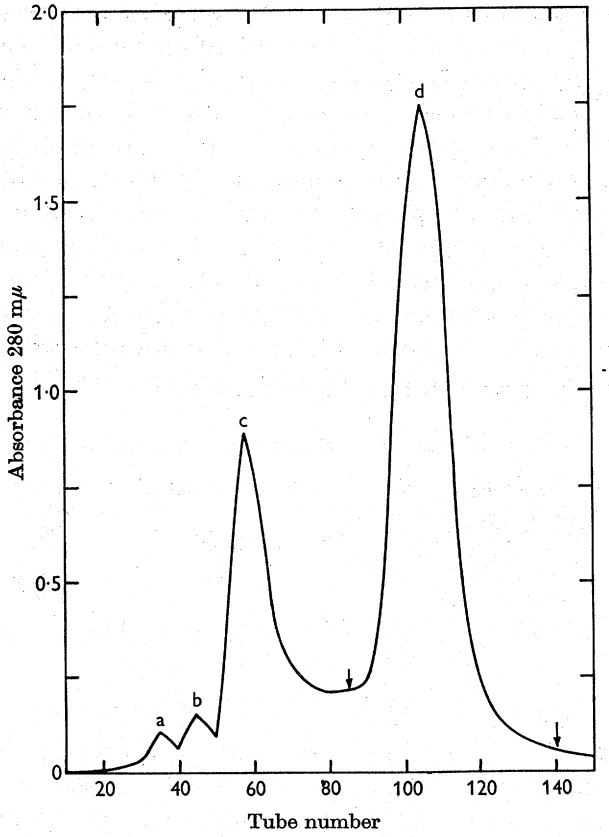

Fig. 1. Whey proteins, unprecipitated by 20 % (w/v) Na_2SO_4, chromatographed on carboxymethylcellulose. Ascending pH gradient at 0·02 ionic strength; 11 ml fractions collected. Peak *d* is β-lactoglobulin and contains both the A and B variant.

Table 5. *Electrophoretic mobilities of the fractions obtained by chromatography of whey proteins on carboxymethylcellulose*

Peak	Tube number	Descending mobility, cm^2/volt-sec × 10^5	Identification
a	30–38	−4·64	Unknown
b	41–48	−5·9 (40 %)	Probably blood serum albumin
		−3·9 (60 %)	Unknown
c	52–67	−4·5 (90 %)	α-Lactalbumin
		−8·3 (10 %)	Unknown
d	85–140	−6·3	β-Lactoglobulins A and B

Kalan, Greenberg & Walter, 1964) this approach is successful. The technique finally arrived at was as follows: whole milk from a heterozygous animal (A/B) was warmed to 40 °C and 200 g/l of anhydrous Na_2SO_4 was added to precipitate the fat, casein, and the immune globulins, as described by Aschaffenburg & Drewry (1957*a*). About 200 ml of the clear greenish filtrate was dialysed against distilled water until free of colour and salt and then against an excess of 0·02 ionic strength NaAc buffer, pH 4·60. In some instances a portion of the α-lactalbumin precipitated at this stage and was removed by centrifugation. The clear solution was applied to a 1·9 × 13 cm column of CMC previously equilibrated with the buffer, and rinsed on with 50–100 ml buffer.

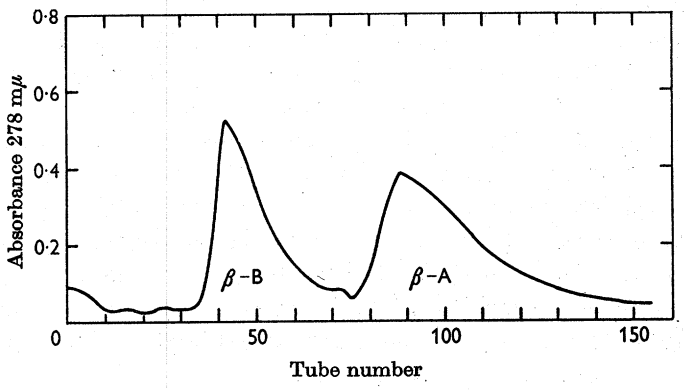

Fig. 2. Peaks of β-A and β-B lactoglobulin obtained by applying the β-lactoglobulin fraction from a carboxymethylcellulose column (Peak *d*, Fig. 1) to a diethylaminoethylcellulose column and eluting with acetate buffer at 0·02 ionic strength using a descending pH gradient. 11 ml fractions collected.

Table 6. *Proportion of β-A in β-lactoglobulin AB from individual Holstein cows*

Animal number	β-lactoglobulin A, % total β-lactoglobulin
3082	52, 51*
3407	54, 56*
3827	57
3806	54
3858	57

* Two samples of the milk of each of these cows were studied.

No u.v. absorbing material was eluted. A continuous pH gradient was made in a closed mixing vessel containing 250 ml of the pH 4·60 buffer, fed by a reservoir containing 1800 ml 0·02 M-NaAc, adjusted to pH 6–6·5 with a few drops of acetic acid. A flow rate of 150 ml/h was easily obtained and a typical elution pattern is shown in Fig. 1. The major peaks obtained were separated and their electrophoretic mobility determined at pH 8·7 in 0·05 ionic strength veronal buffer, as shown in Table 5.

Peak *d* was the β-lactoglobulin peak, containing both the A and B variants, which are not resolved upon Tiselius electrophoresis at pH's near 8·6 (Timasheff & Townend, 1960). The pH of the pooled material under this peak was between 5·2 and 5·4. This entire cut (shown by the arrows on Fig. 1) was then applied to a DEAE column

(1·9 × 13·0 cm), previously equilibrated with a pH 5·2, 0·02 ionic strength NaAc buffer. The β-lactoglobulins are quantitatively retained on this column. A downward pH gradient was applied. A constant volume mixing vessel containing 1100 ml pH 5·2 acetate buffer, 0·02 ionic strength was used with a reservoir containing pH 4·5 buffer of the same ionic strength. The β-lactoglobulins elute as shown in Fig. 2 with better than 95% recovery. As both β-A and β-B have identical absorbancies (Tanford & Nozaki, 1959) the integrated areas under the peaks may be used for quantitation. This was checked using synthetic mixtures of recrystallized β-A and β-B, and results were as follows—%A: 85, found 86; 50, found, 52; 40, found, 37; 15, found 18. Milks from 5 heterozygous animals were analysed by this technique, and the results are given in Table 6.

Within the significance of the analyses, it seems that β-A and β-B are produced in almost equal amounts by heterozygous animals. This result is in disagreement with Lontie, van Goethem, DeWeer & Préaux (1964) who found by agar electrophoresis at pH 8·6, followed by staining, that β-A is produced in much greater amount than β-B. This latter technique should be interpreted with caution, however, since β-A and β-B have almost identical free electrophoretic mobilities at this pH (Timasheff & Townend, 1960) and the effectiveness of the separations obtained on fixed media (Aschaffenburg & Drewry, 1955; Lontie *et al.* 1964) may be due to the differences in rate, and possibly extent, of denaturation during the electrophoresis. Gough & Jenness (1962) have shown that there is a difference in rate of denaturation of β-A and β-B in the range 67–75 °C.

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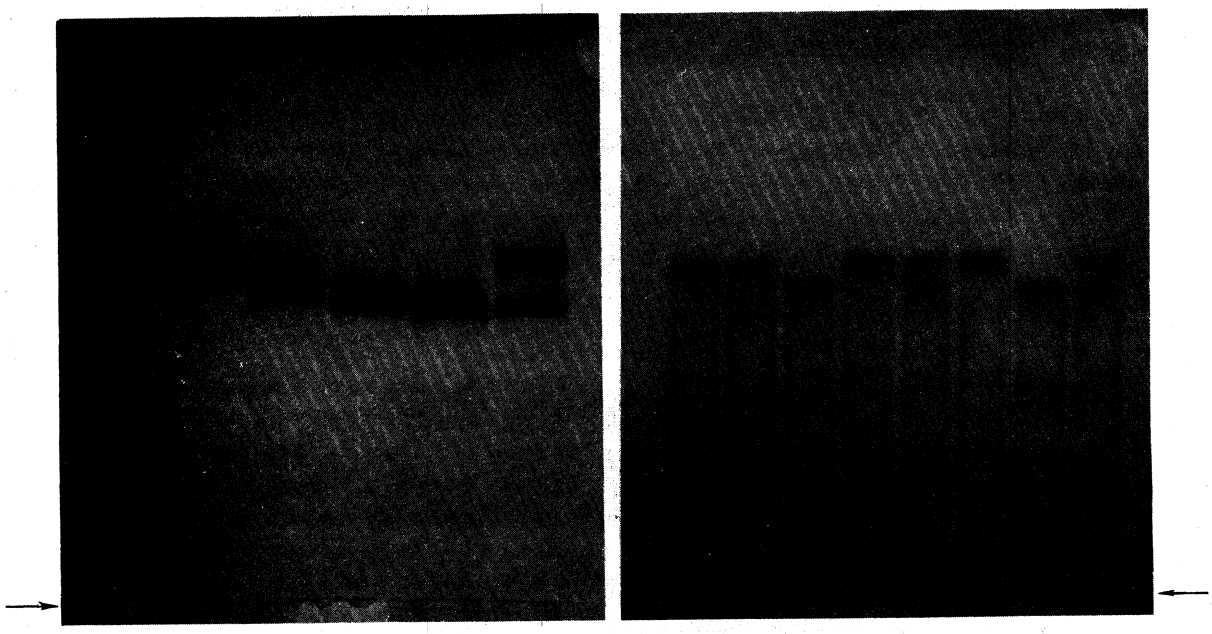
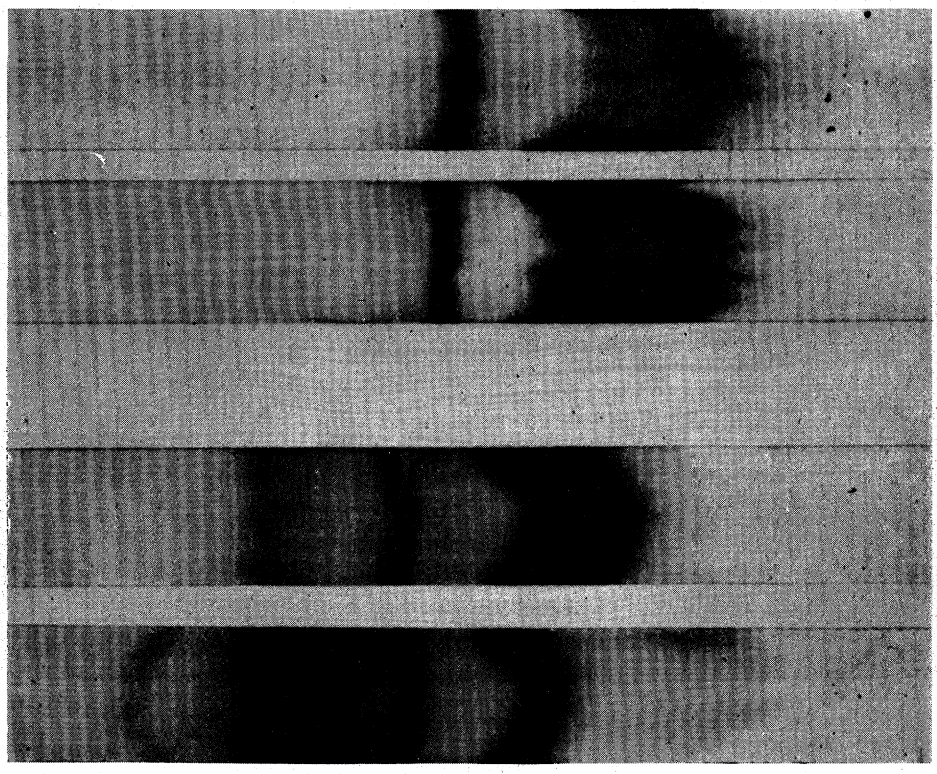


(*a*) (*b*)


(a)
(b)
(c)
(d)

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EXPLANATION OF PLATES

PLATE 1

Polyacrylamide gel electrophoresis of β-lactoglobulins. (*a*), Six possible combinations (types) obtained with recrystallized preparations of β-A, β-B and β-C; left to right A, AB, B, BC, C and AC; (*b*), routine typing run obtained with skim-milk from individual cows; left to right the β-lactoglobulin types are A, AB, B, A, AB, A, B, and AB.

PLATE 2

Paper electrophoresis patterns of milk proteins. (*a*), 20 % (w/v) Na_2SO_4-soluble proteins from an AB cow; (*b*), 20 % (w/v) Na_2SO_4-soluble proteins from castrated male 4120; (*c*), whole whey from an AB cow; (*d*), whole whey from castrated male 4104. Line of application at left edge of Plate.